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Differences in innate immune responses correlate with differences in murine susceptibility to *Chlamydia muridarum* pulmonary infection

Xiaozhou Jiang, Caixia Shen, Hong Yu, Karuna P. Karunakaran and Robert C. Brunham

British Columbia Centre for Disease Control, University of British Columbia, Vancouver, BC, Canada

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Correspondence: R. C. Brunham, University of British Columbia Centre for Disease Control, 655 West 12th Avenue, Vancouver, BC, Canada V5Z 4R4. Email: robert.brunham@bccdc.ca
Senior author: Robert C. Brunham

Summary

We investigated the phenotypic basis for genetically determined differences in susceptibility and resistance to *Chlamydia muridarum* pulmonary infection using BALB/c and C57BL/6 mice. Following *C. muridarum* intranasal inoculation, the intensity of infection was very different between BALB/c and C57BL/6 beginning as early as 3 days post-infection. Intrapulmonary cytokine patterns also differed at early time-points (days 2 and 4) between these two strains of mice. The early recruitment of neutrophils to lung tissue was greater in BALB/c than in C57BL/6 mice and correlated with a higher number of inclusion forming units (IFU) of *C. muridarum*. At day 12 post-infection, BALB/c mice continued to demonstrate a greater burden of infection, significantly higher lung cytokine levels for tumour necrosis factor-α and interleukin-17 (IL-17) and a significantly lower level for interferon-γ than did C57BL/6 mice. *In vitro*, bone-marrow-derived dendritic cells (BMDCs) from BALB/c mice underwent less functional maturation in response to *C. muridarum* infection than did BMDCs from C57BL/6 mice. The BMDCs of BALB/c mice expressed lower levels of activation markers (CD80, CD86, CD40 and major histocompatibility complex class II) and secreted less IL-12 and more IL-23 than BMDCs from C57BL/6 mice. Overall, the data demonstrate that the differences exhibited by BALB/c and C57BL/6 mice following *C. muridarum* pulmonary infection are associated with differences in early innate cytokine and cellular responses that are correlated with late differences in T helper type 17 versus type 1 adaptive immune responses.

Keywords: adaptive immunity; *Chlamydia muridarum*; innate immunity; rodent; susceptibility

Introduction

The *Chlamydiae* is a phylum of intracellular bacteria with unique phylogenetics. *Chlamydia trachomatis* is the major human aetiological agent responsible for several diseases of importance to public health including trachoma and a variety of sexually transmitted diseases which have marked risks for blindness and infertility.[1] There has been long-standing interest in developing an effective vaccine against *C. trachomatis* infection, but progress has been limited in part because of an incomplete understanding of the nature of protective immunity, infection-induced

Abbreviations: 2-ME, 2-methyl-diethyltryptamine; BMDC, bone-marrow-derived dendritic cell; CD, cluster of differentiation; ConA, Concanavalin A; EB, elementary body; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; GM-CSF, murine granulocyte–macrophage colony-stimulating factor; HK EB, heat-killed *Chlamydia muridarum* EB; IFN-γ, interferon-γ; IFU, inclusion-forming unit; IL, interleukin; IMDM, Iscove's modified Dulbecco's medium; KC/CXCL1, keratinocyte-derived chemokine; MEM, minimum essential medium; MHC, major histocompatibility complex; MIP-2/CXCL2, macrophage inflammatory protein 2; PAMP, pathogen-associated molecular pattern; PBS, phosphate-buffered saline; PE, phycoerythrin; SPG, sucrose–phosphate–glutamic acid buffer; Th, T helper; TLR, toll-like receptor; TNF, tumour necrosis factor.

immunopathology and its underlying molecular and cellular basis.[2–6]

Chlamydia muridarum is a mouse-adapted strain whose genome exhibits high similarity to the *C. trachomatis* genome in terms of gene content and chromosomal synteny.[7] Infection of mice with *C. muridarum* has proved to be surprisingly useful in suggesting models for the immunobiology of *C. trachomatis* infection in humans.[8–10] Research regarding the immunology of murine *C. muridarum* infection has revealed that the resolution of primary and secondary infection is highly dependent on T helper type 1 (Th1) –cell-mediated immune responses.[10–12] The Th1-type cytokines such as interleukin-12 (IL-12) and interferon-γ (IFN-γ) play critical roles in controlling and resolving *C. muridarum* infection.[13–18] Adaptive immune responses such as the Th1 response are built upon early innate cellular and cytokine responses, which shape the developmental and migratory pathway exhibited by effector and memory T cells.[19,20] Recruiting effector cells to sites of infection and amplifying and regulating inflammation are also among the major events orchestrated by the innate immune system. The role of dendritic cells (DCs), natural killer cells, macrophages and neutrophils during the innate phase after *C. muridarum* infection remains largely unknown.[21–25] In particular DCs are likely to be particularly important because they are the major professional antigen-presenting cells crucial for initiation of the adaptive T-cell immune response.[5,26]

Toll-like receptors (TLRs) and other classes of pathogen-associated molecular pattern (PAMP) sensors are the fundamental components of the innate immune response that trigger early recruitment of inflammatory cells.[25,27,28] The roles of PAMPs in the host inflammatory response to *C. muridarum* infection are still under active study and limited evidence shows that TLR-2 may be an important mediator in the innate immune response to *C. muridarum* infection, playing a role in both early production of inflammatory cytokine mediators and in the development of chronic inflammatory pathology.[28] *Chlamydia muridarum* molecules that induce PAMP sensor activation remain undefined although plasmid encoded proteins may be associated with activation of TLR-2.[29]

Heterogeneity in host susceptibility to *C. muridarum* infection in both human and murine systems has been well-documented.[10,30–32] In particular, epidemiological observations suggest that the intensity of the early inflammatory response to *C. trachomatis* infection is correlated with the risk of development of late sequelae.[33] Furthermore, it has been suggested that approximately 40% of the heterogeneity in response to *C. trachomatis* infection has genetic underpinnings. The mechanisms underlying genetic variation in the susceptibility to primary *C. muridarum* infection have been best studied using inbred mouse strains.[9,10,32] Yang *et al.*[10] demonstrated that the faster clearance of *C. muridarum* in C57BL/6 mice than in BALB/c mice was related to Th1 cell cytokine IFN-γ production and consequent adaptive T-cell immunity, and was inversely related to early IL-10 production. However, that report did not study the possible connection between variations in innate and adaptive immunity in relation to host susceptibility to *C. muridarum* infection; nor did it evaluate the potential role for the newest T helper lineage member – Th17 cells – in *C. muridarum* immunobiology. We chose the murine model of respiratory *C. muridarum* infection to explore the phenotypic basis for the genetically determined differences that lead to differential outcomes of *C. muridarum* infection in these two inbred strains of mice. We measured microbial growth kinetics, intrapulmonary local cytokine production and inflammatory cell recruitment at both early and late stages of infection. We also evaluated *in vitro* DC responses following *C. muridarum* infection using bone-marrow-derived DCs (BMDCs) collected from these two strains of mice. The findings provide a molecular and cellular explanation for the genetically determined difference in how BALB/c and C57BL/6 mice handle *C. muridarum* infection.

Materials and methods

Reagents and antibodies

All antibodies used for fluorescence-activated cell sorting (FACS) were from BD Pharmingen (Mississauga, ON, Canada), except fluorescein isothiocyanate (FITC)-conjugated anti-mouse F4/80 from Cedarlane Laboratories (Belington, ON, Canada). Chemokine keratinocyte-derived chemokine (KC/CXCL1), and macrophage inflammatory protein 2 (MIP-2/CXCL2) and mouse IL-23 immunoassay were purchased from R&D Systems (Minneapolis, MN); other cytokines for enzyme-linked immunosorbent assay (ELISA) were purchased from BD Pharmingen. Iscove's modified Dulbecco's medium (IMDM), minimum essential medium (MEM), gentamicin, 2-methyl-diethyltryptamine (2-ME) and sodium pyruvate were purchased from Sigma (St Louis, MO); fetal calf serum (FCS) and L-glutamine were purchased from Gibco (Grand Island, NY) and murine granulocyte–macrophage colony-stimulating factor (GM-CSF) was purchased from R&D Systems.

Chlamydia

Chlamydia muridarum mouse pneumonitis strain Nigg was grown in HeLa 229 cells in MEM containing 10% FCS and 0·5 mg/ml cyclohexamide. The *C. muridarum* live elementary bodies (EBs) were purified from infected HeLa cells by discontinuous density gradient centrifugation as previously described[34] and stored in sucrose–phosphate–glutamic acid buffer (SPG) at −80°. The infectivity of purified EBs was determined by infecting HeLa

monolayers for 24 hr, followed by immunostaining and enumeration of inclusions.

Generation and purification of BMDCs

The protocol for generation and purification of BMDCs used in this study has been described previously.[35] Briefly, bone marrow cells were flushed from the femurs of mice and cultured in 20 ml IMDM supplemented with 10% FCS, 4 mM L-glutamine, 50 μg/ml gentamicin, 0·5 mM 2-ME, 10 ng/ml GM-CSF and 5% IL-4 culture supernatants of hybridoma X63. After 7–8 days of culture, the non-adherent cells were harvested and purified using anti-CD11c magnetic beads (Miltenyi Biotech Ltd, Auburn, CA). Routinely the purity of $CD11c^+$ cells was 95 ± 5% (data not shown) as determined by FACS.

Murine pulmonary infection

Eight-week-old female C57BL/6 and BALB/c mice were purchased from Charles River (St Constant, QC, Canada) and housed in a pathogen-free animal facility. All experimental procedures were in accordance with the guidelines approved by the animal care committee of the University of British Columbia. Animals were inoculated intranasally with live *C. muridarum* EBs as previously described.[36] The mice were monitored daily and their body weights were measured. At the indicated time-points, animals were killed by cervical dislocation and the lungs were aseptically harvested for weight, IFU titration, cytokine determination and processing the phenotyping cells using FACS analysis. Susceptibility to *C. muridarum* infection was assessed by changes in body weight, lung weight and lung bacterial burden.

Quantification of bacterial burden in lung

Confluent monolayers of HeLa 229 cells were prepared in flat-bottom 96-well plates after an overnight culture, and then pre-treated with 100 µl per well Hanks' buffered salt solution (Gibco) containing 30 µg/ml diethylaminoethyl-dextran (Pharmacia Fine Chemicals, Uppsala, Sweden) at room temperature for 20 min followed by washing with SPG. Lung tissues were mechanically homogenized with tissue grinders in 5 ml cold phosphate-buffered saline (PBS) per lung followed by centrifugation at 1000 ***g*** for 10 min at 4° to remove coarse tissue debris. The fresh clarified tissue suspensions were serially diluted in SPG and immediately inoculated 100 μl per well onto HeLa monolayers. Left lung homogenates were divided into aliquots and stored at −80° for cytokine assays. After incubation at 37° in 5% CO_2 for 2 hr, the supernatants were removed from HeLa cells and 100 μl per well MEM containing 1 μg/ml cycloheximide (Sigma) was added. The plates were placed in a cell incubator for 24 hr, after which cells were fixed. Immunostaining was carried out using an in-house prepared mouse anti-EB serum as primary antibody and horseradish peroxidase anti-mouse immunoglobulin G as secondary antibody. The colour was developed using 1 × 3,3-diaminobenzidine tetrahydrochloride (DAB)/metal concentrate in stable peroxide substrate buffer (Pierce, Rockford, IL). The *C. muridarum* inclusions were counted under a microscope at × 200 magnifications.

Analysis of cytokine and chemokine by ELISA

Purified BMDCs generated from C57BL/6 or BALB/c mice were plated into U-bottom 96-well plates at 2×10^5 cells in 200 µl of complete IMDM per well and pretreated with or without live *C. muridarum* EBs at a multiplicity of infection of 3 for 48 hr. Spleens were harvested from C57BL/6 or BALB/c mice individually at day 12 after *C. muridarum* intranasal infection and 2×10^6/ml of single-cell suspensions were cocultured with or without 1×10^5/ml of heat-killed *C. muridarum* EBs (HK EBs) in RPMI-1640 medium containing 10% FCS, 4 mM L-glutamine, 50 μg/ml gentamicin and 0·5 mM 2-ME in 24-well plates for 72 hr. Concanavalin A (ConA, 2 µg/ml) was used as a positive control. The culture supernatants were collected and stored at − 80° until they were tested. Lung homogenates were obtained as described above, thawed then vortexed and spun down before they were tested. Cytokines [IL-12, IL-10, IL-6, IL-17, IFN-γ and tumour necrosis factor-α (TNF-α)] in culture supernatants or lung homogenates were determined by ELISA as previously described.[36] Paired anti-mouse IL-12 (p40/p70) (C15.6, C17.8), IL-10 (JESS-2A5, SXC-1), IL-6 (MP5-20F3, MP5-32C11), IL-17 (TC11-18H10.1, TC11-8H4), IFN-γ and TNF-α monoclonal antibodies for ELISA were used. The chemokines KC/CXCL1 and MIP-2/CXCL2 in total lung homogenates were measured using commercially available ELISAs. Production of IL-23 was tested using Quantikine®(R&D Systems, Minneapolis, MN) mouse IL-23 immunoassay according to the manufacturer's instructions.

FACS analysis

For lung inflammatory cell enumeration, groups of four mice were inoculated intranasally with PBS or 8000 IFU live-EBs resuspended in 40 μl PBS. Animals were killed on day 2 post-infection, and their lungs were perfused through the right ventricle of the heart and harvested for total cell isolation. Lung tissues of each mouse were individually minced with scissors to a fine slurry in 0·5 ml PBS containing 2% FCS and subjected to collagenase digestion at room temperature for 20 min in 5 ml of 1 mg/ml collagenase (Sigma) followed by the addition of 200 μl 0·5 M ethylenediaminetetraacetic acid (EDTA, Gibco) per lung. After incubation at room temperature for 5 min, undigested fragments were dispersed by

drawing the solution up and down through the bore of a 10-ml syringe with an 18-gauge needle and were further homogenized using a 40-μm nylon strainer and the plunger of a 1-ml syringe. Red blood cells were lysed by incubating with 0·5 M ammonium chloride. The suspensions of lung cells were centrifuged at 500 ***g*** at 4° for 5 min and washed twice with FACS buffer (PBS containing 2% FCS and 0·1% sodium azide) before staining. For phenotypic analysis of BMDCs, cells were incubated with media alone or live *C. muridarum* EBs at multiplicity of infection of 3 for 24 hr and collected. After washing with FACS buffer twice, cells were ready for staining. Cell staining was performed as previously described.[35] Analysis was carried out on FACS Caliber (Becton Dickinson, San Jose, CA) using CellQuest software (BD Bioscience, Mississauga, ON, Canada) or FlowJo software (Tree star, Ashland, OR); 2×10^5 lung cells and 1×10^4 BMDCs were counted and $1{\cdot}5 \times 10^4$ lung cells (dots) were shown.

The following monoclonal antibodies were used for cell staining: phycoerythrin (PE)-conjugated anti-mouse CD11c (HL3); PE-conjugated anti-mouse CD49b (DX5); peridinin chlorophyll protein-Cy™5.5-conjugated anti-mouse (BD Pharmingen, Missisauga, ON, Canada) CD11b (M1/70); FITC-conjugated anti-mouse CD80 (16-10A1); FITC-conjugated anti-mouse CD86 (GL1); FITC-conjugated anti-mouse I-A/I-E (2G9); FITC-conjugated anti-mouse CD40 (HM40-3); FITC-conjugated rat anti-mouse Gr-1 (RB6-8C5); FITC-conjugated anti-mouse CD3 (17A2); FITC-conjugated anti-mouse F4/80 (CL8940F); and FITC-conjugated anti-mouse TLR-2 (6C2).

Statistical analysis

Statistical analysis was conducted using two-tailed unpaired Student's *t*-test for ELISA, FACS analysis, IFU, body weight and lung weight comparisons of each time-point. Repeated measures analysis of variance (RM ANOVA) was used for the changes in body weight over time between the two groups. Differences at the $P < 0{\cdot}05$ level were considered statistically significant.

Results

Susceptibility to *C. muridarum* infection and disease in BALB/c versus C57BL/6 mice

Each group of animals was infected in parallel with *C. muridarum* via intranasal inoculation. Susceptibility was assessed by monitoring weight loss and lung bacterial burden. As shown in Fig. 1, beginning at day 3 BALB/c mice lost significantly more body weight than C57BL/6 mice after infection (Fig. 1a) and at day 12 post-infection the bacterial burden in the lungs of BALB/c mice remained significantly greater than in C57BL/6 mice ($182{\cdot}6 \pm 28{\cdot}4 \times 10^6$ versus $75{\cdot}2 \pm 34{\cdot}4 \times 10^6$ IFU)

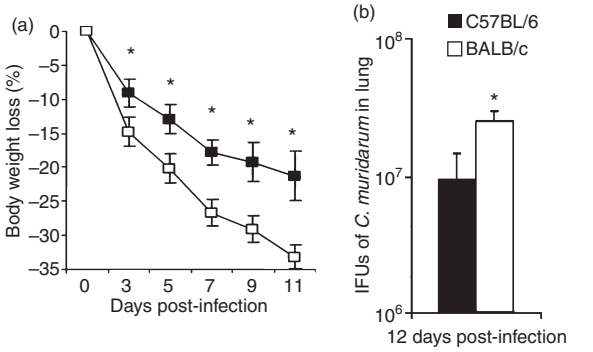

Figure 1. Susceptibility of BALB/c mice and C57BL/6 mice following *Chlamydia muridarum* infection. Groups of eight mice were intranasally inoculated with 2000 inclusion-forming units (IFU) of *C. muridarum* and body weight changes were monitored every 2 days (a). Mice were killed at day 12 post-infection and IFU in lung tissue were titrated as described in the *Materials and methods* (b). The data represent the mean ± SD from eight individual mice. One of three independent trials with similar results is shown. *$P < 0{\cdot}05$.

(Fig. 1b). The changes in body weight were also compared over time for the two groups by two-way RM ANOVA; body weight loss in BALB/c was significantly greater than C57BL/6 ($P < 0{\cdot}001$). Additionally, BALB/c mice exhibited more clinical signs of disease, as indicated by lethargy, ruffled fur and laboured breathing, than did C57BL/6 mice. The results confirm previous reports that BALB/c mice are more susceptible to *C. muridarum* pulmonary infection than C57BL/6 mice.

To investigate whether these differences correlate with innate immunity, we tested in separate experiments the early responses of mice following *C. muridarum* infection. Body weights were measured daily until day 4 post-infection. Beginning day 3 post-infection BALB/c mice showed significantly greater loss of body weight than C57BL/6 mice (Fig. 2a). Change in lung weight is an established index of disease severity in murine models following pulmonary infection.[37] As shown in Fig. 2(b), lung weights exhibited no difference at day 2 post-infection but by day 4 they were significantly greater in BALB/c mice than in C57BL/6 mice, indicating more severe infection in BALB/c mice than in C57BL/6 mice. Lung titration of *C. muridarum* revealed significantly higher IFU in lung homogenates of BALB/c than C57BL/6 mice at both day 2 ($14{\cdot}2 \pm 1{\cdot}3 \times 10^6$ versus $4{\cdot}3 \pm 1{\cdot}5 \times 10^6$) and day 4 ($269{\cdot}5 \pm 36{\cdot}3 \times 10^6$ versus $155{\cdot}5 \pm 103{\cdot}5 \times 10^6$) after infection (Fig. 2c). These results show that genetic differences in susceptibility to *C. muridarum* lung infection are demonstrable at early stages of infection and probably reflect differences in innate response.

Early and late local cytokine profiles in response to *C. muridarum* infection

In the early stage of infection, the balance among cytokines can be very important in containing infection and

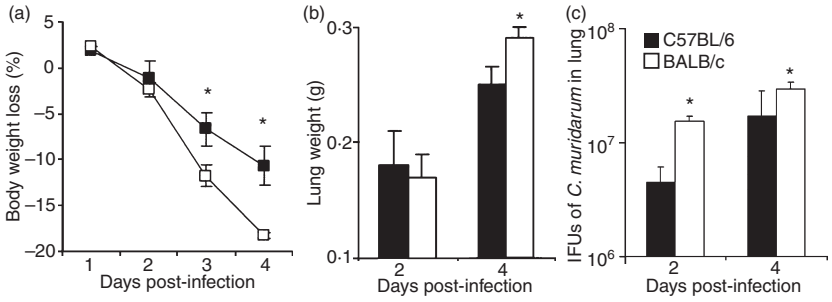

Figure 2. Early stage susceptibility of BALB/c mice and C57BL/6 mice following *Chlamydia muridarum* infection. Groups of eight mice were intranasally inoculated with 8000 inclusion-forming units (IFU) of *C. muridarum* and body weight changes were monitored daily up to 4 days (a). Mice were killed at days 2 or 4 post-infection and the lungs were weighed (b). Infectivity was assessed by lung IFU titration at days 2 and 4 (c). The data represent the mean ± SD from eight individual mice. One of three independent trials with similar results is shown. *$P < 0{\cdot}05$.

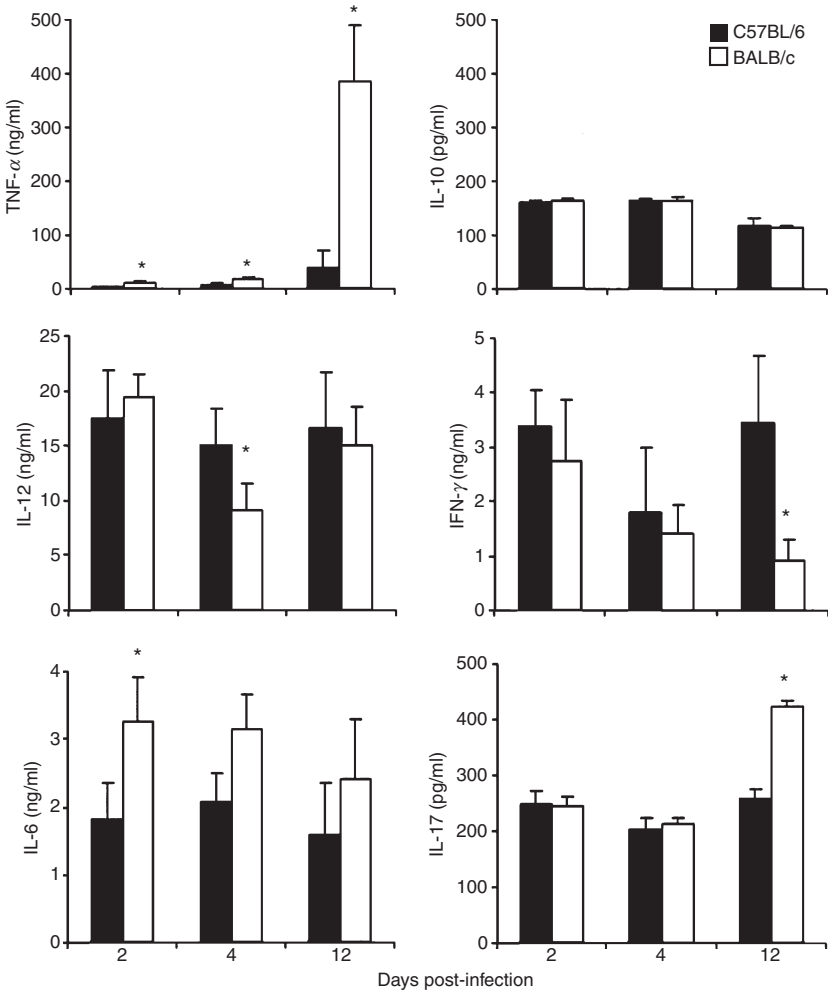

Figure 3. Cytokine secretions in lung tissue of BALB/c mice versus C57BL/6 mice after infection with *Chlamydia muridarum*. Groups of eight mice for each strain were inoculated intranasally with 8000 inclusion-forming units (IFU; for days 2 and 4 infection) or 2000 IFU (for day 12 infection) of *C. muridarum*. At days 2, 4 and 12 post-infection, lung homogenates were prepared for lung IFU titrations and for assays of cytokine levels by enzyme-linked immunosorbent assay. The data represent the mean ± SD from eight individual mice. One of three independent trials with similar results is shown. *$P < 0{\cdot}05$.

in triggering T-cell and B-cell developmental pathways. Therefore, we compared the production of several important pro-inflammatory cytokines including TNF-α, IL-6, IL-10, IL-12, IL-17 and IFN-γ in the lungs at both early (days 2 and 4) and later (day 12) stages of infection. As shown in Fig. 3, BALB/c produced significantly higher levels of TNF-α in lung homogenates than C57BL/6 mice on both days 2 and 4 after *C. muridarum* infection as determined by ELISA. Levels of IL-6 showed a pattern similar to TNF-α although the differences were statistically significant only at day 2 post-infection. C57BL/6 mice showed significantly higher levels of IL-12

production at day 4 post-infection than BALB/c mice. There were no statistically significant differences in IFN-γ, IL-10 and IL-17 levels at the early time-points. By day 12 post-infection BALB/c mice demonstrated significantly higher TNF-α and lower IFN-γ levels than C57BL/6 mice. Notably, intrapulmonary IL-17 levels were significantly greater in BALB/c mice than in C57BL/6 mice at day 12 post-infection. These data demonstrate that different cytokine profiles occur both early and late in response to *C. muridarum* infection in BALB/c versus C57BL/6 mice.

C. muridarum cytokine responses in BALB/c and C57BL/6 mice

To confirm that the differences of IFN-γ and IL-17 levels at day 12 post-infection in BALB/c versus C57BL/6 mice are correlated with late adaptive Th17 and Th1 immune responses, we investigated *C. muridarum* cellular cytokine responses. Splenocytes were harvested at day 12 from mice infected with 2000 IFU *C. muridarum* and cultured *in vitro* with or without HK EB for 72 hr. The *C. muridarum* IFN-γ and IL-17 responses were measured by ELISA. As shown in Fig. 4, BALB/c mice demonstrated a significantly lower *C. muridarum* IFN-γ response (8·3 ± 2·4 ng/ml versus 43·3 ± 12·6 ng/ml) and higher *C. muridarum* IL-17 response (100·1 ± 13·9 pg/ml versus 47·6 ± 3·5 pg/ml) than C57BL/6 mice. For the positive control, ConA (0·2 μg/ml) was used (9·1 ± 1·4 ng/ml versus 15·2 ± 4·3 ng/ml for IFN-γ and 159·0 ± 19·6 pg/ml versus 112·9 ± 13·3 pg/ml for IL-17) and showed no significant difference. Higher Th17 and lower Th1 cytokine responses in splenocytes are consistent with higher IL-17 and lower IFN-γ levels directly measured in the lungs of BALB/c compared with C57BL/6 mice.

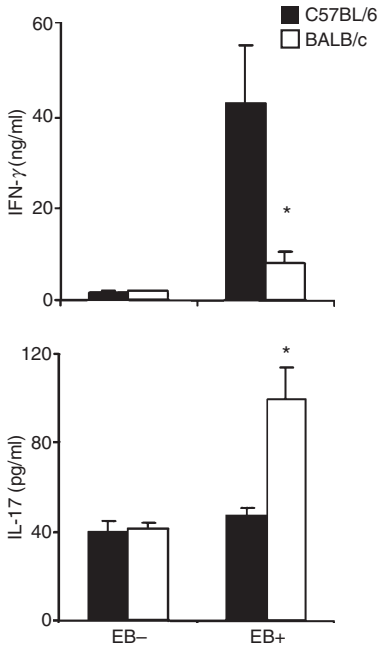

Figure 4. Cytokine production by splenocytes in response to *Chlamydia muridarum* stimulation. Groups of nine mice were inoculated intranasally with 2000 inclusion-forming units (IFU) of *C. muridarum* and splenocytes were prepared at day 12 followed by stimulation with or without heat-killed *C. muridarum* elementary bodies (EBs) for 72 hr. The level of *C. muridarum* responsive interferon-γ (IFN-γ) and interleukin-17 (IL-17) in splenocyte culture supernatants was analysed by enzyme-linked immunosorbent assay. The data represent the mean ± SE from nine individual mice. *$P < 0{\cdot}05$.

Early cellular recruitment and chemokine production in response to pulmonary *C. muridarum* infection

Rapid cellular recruitment and exudative responses triggered by pro-inflammatory cytokines are hallmarks of the acute inflammatory response. Accordingly, we examined the phenotype of lung cell recruitment by FACS in response to intranasal *C. muridarum* infection. BALB/c and C57BL/6 mice were intranasally infected with *C. muridarum* or with PBS alone and lung cells were analysed on day 2 by double staining of surface markers. The counts of total lung cells were $3{\cdot}3 \pm 1{\cdot}2 \times 10^6$ cells/lung in BALB/c versus $4{\cdot}2 \pm 0{\cdot}9 \times 10^6$ cells/lung in C57BL/6 before infection and $8{\cdot}3 \pm 1{\cdot}3 \times 10^6$ cells/lung in BALB/c versus $8{\cdot}6 \pm 1{\cdot}0 \times 10^6$ cells/lung in C57BL/6 after infection. Surface markers evaluated include $DX5^+$ $CD3^-$ for NK cells, $Gr1^+$ $CD11b^+$ for neutrophils, $CD11b^+$ $CD11c^+$ major histocompatibility complex class II $(MHC\ II)^+$ for myeloid DCs, and $F4/80^+$ $CD11b^+$ for macrophages. Compared with lung cells from PBS-inoculated mice, rapid infiltration with neutrophils, DCs and macrophages was observed in both strains of mice following infection (Table 1). Neutrophils, however, were more significantly recruited in BALB/c mice than in C57BL/6 mice in response to intranasal *C. muridarum* infection. Fewer DCs and macrophages were detected

Table 1. Lung cell recruitment was analysed by mean percentage ± SD of each cell population in total lung cells by fluorescence-activated cell sorting in C57BL/6 and BALB/c mice after 2 days intranasal infection with 8000 inclusion-fomring units of *Chlamydia muridarum*

	Uninfected		Infected	
	C57/BL6	BALB/c	C57/BL6	BALB/c
Natural killer cell	16·5 ± 0·5	13·7 ± 0·4	16·9 ± 1·9	17·9 ± 1·7
Myeoid denritic cell	0·1 ± 0·1	0·2 ± 0·01	**2·9 ± 0·5**	**1·5 ± 0·05***
Neutrophil	2·0 ± 0·03	3·3 ± 0·3	**25·8 ± 2·1**	**36·8 ± 3·7***
Macrophage	3·3 ± 0·5	1·6 ± 0·2	**16·4 ± 1·2**	**8·4 ± 0·5***

Three individual mice in each group. One representative experiment of two independent trials with similar results is shown. Numbers in bold represent statistically significant values when C57/BL6 and BALB/c were compared.
*$P < 0{\cdot}05$.

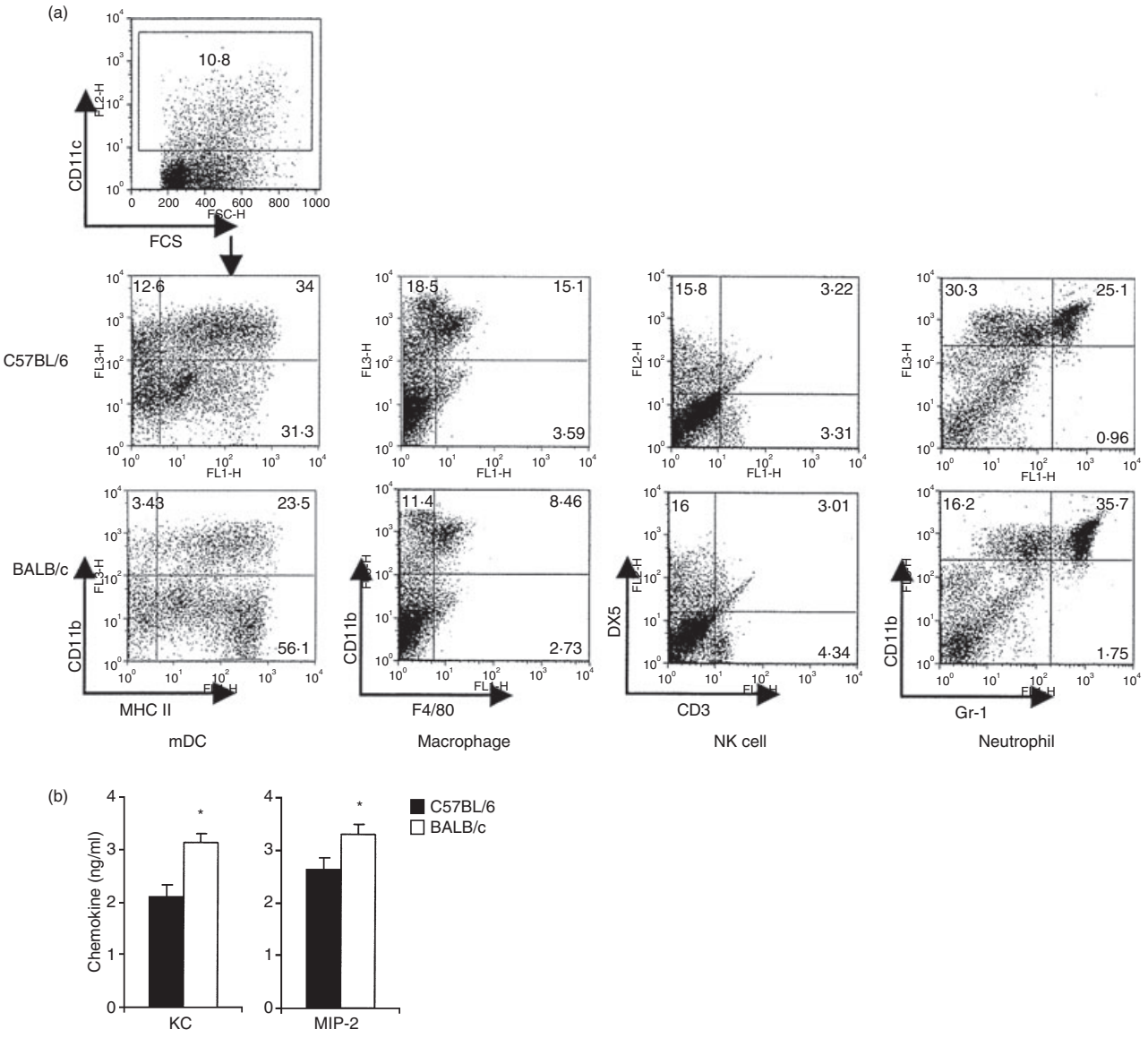

Figure 5. Cell recruitment and neutrophil chemokine production in lung tissue of BALB/c and C57BL/6 mice after infection with *Chlamydia muridarum*. Groups of three mice were inoculated intranasally with 8000 inclusion-forming units (IFU) of *C. muridarum* and day 2 post-infection lungs were harvested, single cell suspensions were created and cell types were enumerated by fluorescence-activated cell sorter analysis. Two independent experiments were carried out. Dot-plot from one representative mouse shows double staining of surface markers for lung inflammatory cells (a). Chemokine secretion in lung tissue of BALB/c mice and C57BL/6 mice at day 2 after infection with *C. muridarum*. Lung homogenates were the same samples prepared for Fig. 3. The chemokines in lung homogenates were analysed by enzyme-linked immunosorbent assay. The data represent the mean ± SE from eight individual mice. One of three independent trials with similar results is shown. $*P < 0{\cdot}05$ (b).

in BALB/c mice than in C57BL/6 mice. The recruitment of NK cells was similar between these two strains of mice (Fig. 5a, Table 1).

To explore potential causes for the early increased influx of neutrophils, we tested the concentrations of KC and MIP-2 chemokines in lung homogenates by ELISA. The results demonstrated that both of these neutrophil chemokines were significantly higher in BALB/c than C57BL/6 mice at day 2 after *C. muridarum* infection (Fig. 5b).

In vitro activation of BMDCs derived from BALB/c and C57BL/6 mice after infection with *C. muridarum*

We were particularly interested in DCs as critical participants in the innate response by virtue of their ability to link innate and adaptive immunity. The endocytosis of *C. muridarum* by BMDCs from BALB/c and C57BL/6 mice was tested and exhibited no significant difference as measured by IFU (data not shown). To evaluate the activation response of BMDCs following *in vitro* infec-

tion with *C. muridarum* from these two strains of mice, we determined the expression of cell surface molecules such as CD40, CD80, CD86, MHC II and TLR-2 on BMDCs followed by determination of secretion levels of selected cytokines. After 24-hr cultures, the cells were collected and subjected to FACS analysis; and after 48 hr of culture, the supernatants from parallel sets of cultures were collected and the cytokines in the media were tested by ELISA. The expression level of activation molecules on BMDCs was estimated from fluorescence intensity. Uninfected resting BMDCs derived from these two strains of mice displayed no difference in expression of CD40, CD80, CD86, MHC II and TLR-2 (data not shown). After *C. muridarum* infection, the expression of CD80, CD86, CD40 and MHC II was less increased on BMDCs derived from BALB/c mice than on those from C57BL/6 (Fig. 6a,b). Expression of TLR-2 was slightly greater on BMDCs derived from BALB/c mice than on those from C57BL/6 mice. We next evaluated the response of DCs to *C. muridarum* infection by comparing the cytokine secretion profiles. As shown in Fig. 6(c), *C. muridarum*-infected BMDCs derived from BALB/c mice secreted lower levels of IL-12 and higher levels of IL-23, IL-6, IL-10 and TNF-α than those derived from C57BL/6 mice. Remarkably these *in vitro* cytokine responses of infected BMDCs closely resemble those observed *in vivo* following pulmonary *C. muridarum* infection.

Discussion

Genetic differences in susceptibility to many infectious diseases including *C. muridarum* have been studied using inbred mouse models.[5,9,10] In this study, we have demonstrated that the genetically determined differences in susceptibility to pulmonary *C. muridarum* infection between BALB/c and C57BL/6 mice start at early time-points following infection during which the innate responses are occurring. The results show that the differences include greater decreases in body weight, larger increases in lung weight and accelerated growth of *C. muridarum in vivo* (Fig. 2) in BALB/c mice compared to C57BL/6 mice. The difference in the extent of body weight loss between BALB/c and C57BL/6 mice was first statistically observed at day 3 (Fig. 2a) with the same trend maintained through to day 12 (Fig. 1a). The greater increase in lung weight from day 2 to 4 post-infection in BALB/c mice as compared with C57BL/6 mice shown in Fig. 2(b) probably indicates more severe pathological changes in the lung and was accompanied by more severe clinical signs of respiratory disease. Study of early cellular infiltration by FACS analysis of lung cells following *C. muridarum* infection revealed a more intense inflammatory reaction in BALB/c than C57BL/6 mice with markedly more infiltrating neutrophils (Table 1).

Differences in susceptibility to *C. muridarum* that occur at early stages in infection are most likely related to the differences in the host innate response.[20] Innate immunity involves a complex network of cytokines, chemokines, plasma proteins and local cellular recruitment.[19] Chemokine KC and MIP-2 are potent neutrophil chemoattractants and their concentrations in lung homogenates at day 2 were higher in BALB/c mice than in C57BL/6 (Fig. 5b). These differences in chemokine concentration may be responsible for the different neutrophil influxes between BALB/c and C57BL/6 mice (Fig. 5a and Table 1).

Many cytokines have been implicated in *C. muridarum* host defence and in this study we compared the cytokine profiles in lung homogenates at days 2, 4 and 12 after infection. The most remarkable difference was observed with TNF-α level. The levels of lung TNF-α were substantially higher in the BALB/c than in C57BL/6 mice at all time-points studied, especially at day 12. Interleukin-6 is another cytokine produced in significant amounts in BALB/c mice at early time-points in the lung in response to *C. muridarum* infection although this IL-6 response was more transient than the TNF-α response. Studies have indicated that TNF-α and IL-6 may play roles in host defence against *C. muridarum* infection, but it remains unclear whether they mediate protective or pathological responses.[38–42] In this study, the higher TNF-α and IL-6 productions in the lung observed in BALB/c mice correlated with enhanced susceptibility to *C. muridarum* at both early and late stages of infection.

Interferon-γ is the cytokine that has been most thoroughly studied in *C. muridarum* protective immunity. Our data show that the IFN-γ production in the lungs of BALB/c and C57BL/6 mice was similar at days 2 and 4 but became statistically different at day 12, which suggests that adaptive T-cell responses are the likely source for IFN-γ during the late phases (Fig. 4). The late difference in IFN-γ secretion between the two strains of mice was temporally correlated with an observed early difference in IL-12 secretion, because the lung IL-12 level in BALB/c mice was significantly lower than in C57BL/6 mice at day 4. The lower IL-12 level in the lungs of BALB/c mice may be related to less recruitment of DCs and macrophages in early infection (Table 1) and lower secretion of IL-12 following DC infection with *C. muridarum* (Fig. 5).

Overall, the lung cytokine assays showed two different response profiles. One pattern observed in BALB/c mice was of high TNF-α and IL-6 levels and low IL-12 production, which was associated with more severe neutrophilic inflammation, poorer clearance of *C. muridarum* and later development of an adaptive Th17 immune response. The other response pattern observed in C57BL/6 mice was associated with higher IL-12 and lower TNF-α and IL-6 secretion, which was associated with less lung inflammation, more rapid clearance and the later development of Th1 IFN-γ-mediated immunity.

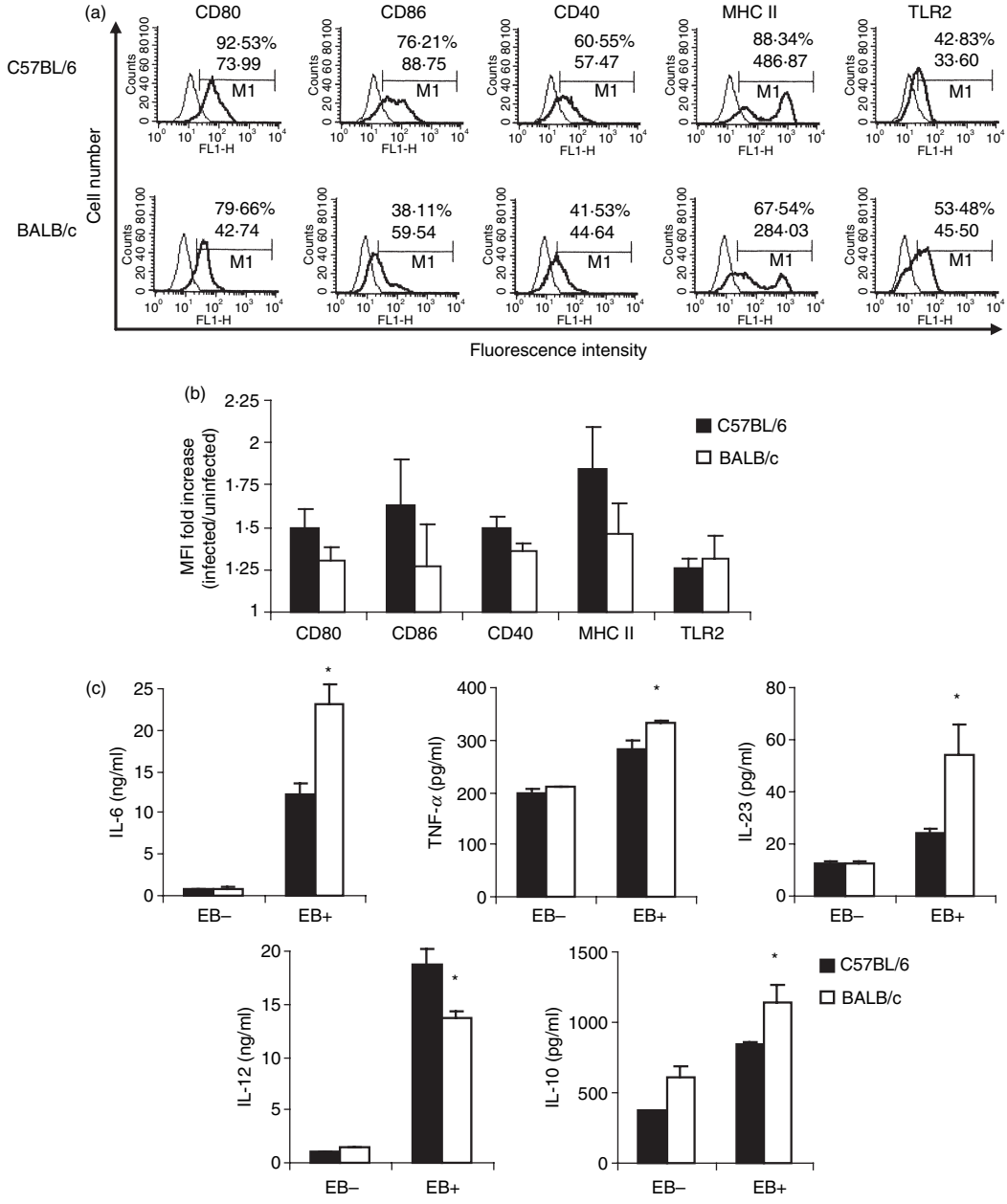

Figure 6. Surface phenotype and cytokine secretion of cultured dendritic cells (DCs) derived from BALB/c mice and C57BL/6 mice. Bone-marrow-derived dendritic cells (BMDCs) were generated and purified from C57BL/6 and BALB/c mice as described in the *Materials and methods*. Cells were infected with live *Chlamydia muridarum* elementary bodies at a multiplicity of infection of 3 for 24 hr and collected for staining of costimulatory molecule expression [CD80, CD86, CD40, major histocompatibility complex class II (MHC II) and Toll-like receptor 2 (TLR2)] by fluorescence-activated cell sorting (FACS). The thin-line histograms represent unstained cells as controls and bold-line histograms represent stained cells. Numerical values depict the percentage of positive cells and fluorescence intensity of relevant surface molecules. Data are representative of three independent experiments with similar results (a). Fold increase of surface molecular expression on BMDCs after *C. muridarum* stimulation represent mean ± SE ($n = 9$) of three separate experiments (b). After 48 hr incubation with or without *C. muridarum*, culture supernatants were collected for enzyme-linked immunosorbent assay cytokine test. The data represent the mean ± SD of three separated experiments. $^{*}P < 0{\cdot}05$ (c).

A novel finding in this study is the higher lung IL-17 levels in BALB/c mice than in C57BL/6 mice at day 12 after *C. muridarum* infection. Interleukin-17 is a recently recognized pro-inflammatory cytokine produced by Th17 cells.[43] These Th17 cells appear to be critical T-cell immune effectors to extracellular pathogens at the mucosal surface and have been implicated in autoimmunity or immunopathology. The role of IL-17 in *C. trachomatis* immunobiology is not yet clear. As IL-6 is one of the key effector cytokines involved in triggering the

Th17 cell developmental pathway, the early high levels of IL-6 in BALB/c mice may play a role in promoting the adaptive Th17 immune pathway,[44] which may in turn have contributed to the stronger neutrophilic inflammatory response observed in BALB/c mice. Corresponding with the higher lung IL-17 levels at day 12 post-infection, *C. muridarum*-responsive IL-17 production by splenocytes from *C. muridarum*-infected mice was also higher in BALB/c mice than in C57BL/6 mice (Fig. 4). Importantly these differences in cytokine responses between the two strains of mice were seen only with *C. muridarum* EBs but not with ConA. Taken together, these data suggest that the genetic differences in innate immune responses in these two strains of mice shape the divergent T-cell adaptive immune responses and thereby determine infection kinetics and disease outcome.

Dendritic cells stand centre stage in linking innate to adaptive T-cell responses.[45,46] Some studies have indicated that BMDCs from different genetic backgrounds show different levels of activation, endocytosis and cytokine production after microbial stimulation.[47–50] It was therefore of great interest that when DCs from each mouse strain were infected with *C. muridarum in vitro*, distinct maturation patterns and cytokine secretion profiles were observed. Although no differences between resting BMDCs from the two mouse strains were observed, after *in vitro C. muridarum* infection the purified BMDCs derived from BALB/c mice demonstrated a smaller increase in expression of activation markers, including CD80, CD86 and MHC II, than did BMDCs from C57BL/6 mice (Fig. 6a,b). The BMDCs derived from BALB/c mice secreted lower levels of IL-12 and higher levels of IL-6, IL-10 and TNF-α *in vitro* compared with BMDCs from C57BL/6 mice following *C. muridarum* infection. Furthermore, DCs from BALB/c mice secreted more IL-23 than DCs from C57BL/6 after infection with *C. muridarum* (Fig. 6c). As IL-23 has an important role in the stabilization of Th17 cells, the higher IL-23 secretion from DCs may be responsible for the higher Th17 responses in BALB/c mice. The different responses of infected BMDCs may directly or indirectly alter the innate and adaptive immune responses. Darville *et al.*[28] demonstrated that TLR-2 is an important mediator in the innate immune response to *C. trachomatis* infection and that it is essential for development of oviduct pathology in *C. muridarum* genital tract infection. Of potential interest is the finding that the TLR-2 expression on BMDCs from BALB/c mice is slightly increased compared with that on BMDCs from C57BL/6 mice after *in vitro* stimulation with *C. muridarum*. The impact of TLR-2 expression by DCs *in vivo* on the inflammatory response needs further study. Taken together, these findings suggest that DCs may be the target cell in which the genetically defined differences in *C. muridarum* immunobiology between BALB/c and C57BL/6 mice are phenotypically expressed. Overall, these findings in the murine *C. muridarum* model should be useful in defining new immunoepidemiological studies of human *C. trachomatis* infection.

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Disclosures

The authors have no financial conflict of interest.

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